Biophysical Chemistry 22 (1985) 17–26
Elsevier

BPC 00920

INTERACTION OF PAIRED HOMOLOGOUS SERIES OF DIACRIDINES AND TRIACRIDINES WITH DEOXYRIBONUCLEIC ACID

William A. DENNY [a], Graham, J. ATWELL [a], Gayle A. WILLMOTT [b] and Laurence P.G. WAKELIN [b,*]

[a] *Cancer Research Laboratory, School of Medicine, University of Auckland, Private Bag, Auckland, New Zealand, and* [b] *Experimental Chemotherapy Unit, Cancer Institute, 481 Little Lonsdale Street, Melbourne, Victoria 3000, Australia*

Received 7th November 1984
Accepted 7th January 1985

Key words: Triacridine; Diacridine; Sonicated DNA; Circular DNA; Intercalation; Viscometry

Viscometric measurements using covalently closed circular DNA and sonicated rod-like DNA fragments were performed to investigate unwinding and extension of the DNA helix associated with binding of paired homologous series of diacridines and triacridines. The maximum interchromophore distance for members of the diacridine series spans from 15.1 to 27.5 Å, permitting the largest of these ligands to cover up to 4 or 5 base-pairs, allowing for helical twist and local unwinding in a bisintercalated complex lacking severe bending or kinking of the DNA backbone. Helix unwinding angles and increments in DNA contour length are characteristic of bifunctional reaction for all the diacridines studied, the DNA lattice appearing to saturate with one ligand molecule bound per 4 base-pairs. The triacridines, whose maximum end-to-end interchromophore distances are the same as those of their paired diacridines, have maximum central-to-terminal interchromophore distances covering the range 7.5–13.8 Å and thus have the potential to form trisintercalated complexes with one or two base-pairs sandwiched between each chromophore. However, helix extension and unwinding parameters for the triacridines are similar to those of the diacridines, and we find no evidence of a transition from bifunctional to trifunctional reaction as the homologous series is ascended. In general, the binding site size appears to be 5 base-pairs for the triacridines. The stereochemical requirements for trisintercalation of triacridines are discussed with reference to the present findings and to the work of others.

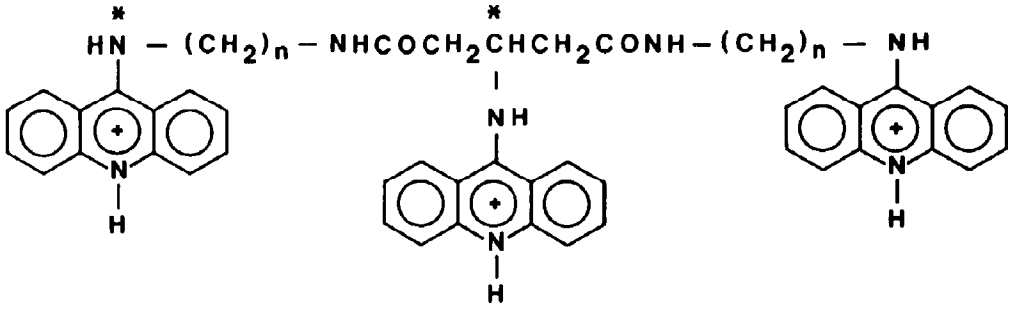
1. Introduction

Several research groups interested in the medicinal application of DNA intercalating agents have focussed their attention on the development of bifunctional ligands [1–5]. The principal motivation is that bisintercalation affords the opportunity to improve specificity for DNA per se, as well as nucleotide sequence selectivity, as a result of the larger association constants and binding site sizes expected for drugs of this type [6,7]. Most biological studies have centred upon the cytotoxic and antitumour activity of these agents and in some systems bifunctional ligands have demonstrated much enhanced efficacy compared to their parental mononuclear drugs [3,5,8–10]. Studies of the structural requirements for bifunctional intercalation amongst dimers of 9-aminoacridine have established that simple ligands, in which the acridines are unsubstituted and the linker chain is composed entirely of methylene groups, can form bisintercalated complexes in which a single base-pair is sandwiched between the intercalated acridine chromophores [11]. However, if the acridine is substituted in the 2 or 3 position, so that the molecule is extended along its major axis [12], or the linker chain is made more rigid by the incorporation of amide groups [13], then bisintercalation is not observed until the linker is sufficiently long to span two base-pairs in the drug-

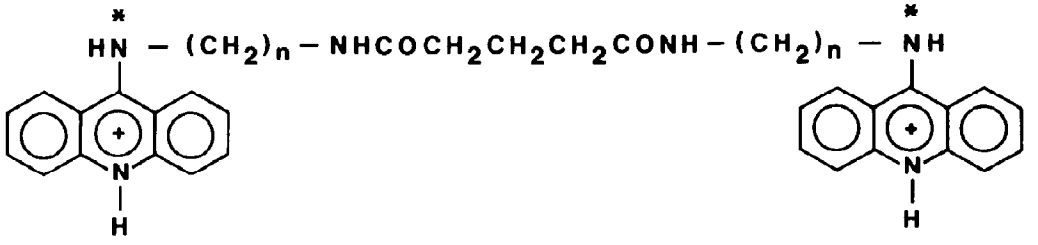
* To whom correspondence should be addressed.

DNA complex. It is generally believed, although not universally accepted [3], that the biological activity of these agents derives from their ability to disrupt the template functions of DNA by competing for polymerase and gene-regulatory protein-binding sites on the polynucleotide. This mode of action would suggest that further improvements in selective toxicity may be obtained with larger polyfunctional ligands that have the capacity to extend over a greater number of nucleotides, and thus the potential to bind with enhanced specificity to longer nucleotide sequences. One approach is to use trisintercalating agents and the fundamental question is then the nature of the linker chain and the chromophore spacing required to promote trifunctional reaction.

In a preliminary communication two of us have reported that a triacridine comprising three 9-aminoacridine moieties separated by a distance sufficient to encompass only a single base-pair in each arm of a fully intercalated DNA complex binds with all three chromophores so intercalated [14]. This conclusion was based upon measurements of helix unwinding angles and visible ex-



triacridines



diacridines

Fig. 1. Structural formulae of the diacridines and triacridines. *n* has the values 2–7 in each series.

tinction coefficients of bound ligands. A structurally similar triacridine has been synthesised by Gaugain et al. [15] in which the linkage is long enough to span two nucleotide pairs between each chromophore. When assessed by its capacity to unwind DNA this compound also appears to trisintercalate, but this finding is not corroborated by the increase in DNA contour length accompanying binding which is characteristic of bifunctional reaction [2,15]. The interaction with DNA of a third triacridine, in which the 9-aminoacridine chromophores are linked in a different manner to the previous examples, has been investigated by Hansen et al. [16], who conclude on the basis of helix extension and visible extinction coefficient measurements that it too trisintercalates. Its chemical structure suggests there is room for only a single base-pair between each bound chromophore, although Hansen et al. [16] make no comment on the nature of its DNA complex. A characteristic common to all three studies described above is that only single examples of triacridine ligands have been investigated, thereby precluding generalisations about structure-function relationships.

We report here detailed viscometric studies designed to evaluate the mode of binding of paired homologous series of triacridines and their companion diacridines lacking the middle chromophore, in which the central-to-terminal interchromophore distance for the fully extended triacridines spans the range necessary to cover one and two base-pairs in a trisintercalated complex (see fig. 1 for structures). Assessments of functionality have been made by comparing helix unwinding angles and increments in DNA contour length accompanying binding of the triacridines and diacridines with those found for 9-aminoacridine, as determined by the ability of the ligands to remove and reverse the supercoiling of covalently closed circular DNA and to increase the viscosity of sonicated rod-like fragments of calf thymus DNA [11]. We find no evidence for a transition from bifunctional to trifunctional reaction as the homologous series is ascended, a result which is at variance with the conclusions of the preliminary report for a pair of the ligands described here [14]. Possible reasons for this discrepancy and the geometrical constraints for trisintercalation within this series of compounds are discussed.

2. Materials and methods

2.1. Materials

Ethidium bromide was obtained from Sigma Chemical Co. (U.S.A.) and 9-aminoacridine prepared by standard synthetic procedures. The di- and triacridines were synthesised and purified as described elsewhere [14]; their molecular formulae, formula weights, visible extinction coefficients and maximum internuclear distances are given in table 1. In the interest of clarity these compounds are referred to in the text as C_n diacridine and C_n triacridine where n is the number of carbon atoms in the connecting methylene chains (see fig. 1). Where possible all ligands were dissolved in buffer at a concentration of 1 mM and stock solutions stored frozen in the dark at $-20°C$. The C_6 and C_7 di- and triacridines, being of more limited solubility, were dissolved to a concentration of 500 μM. The buffer used throughout (designated 0.01 SHE) contained 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, 10 μM EDTA and 9.4 mM NaCl dissolved in ultrapure water from a Barnstead Nanopure System. It was adjusted to pH 7.0 at 20°C with NaOH, the resultant ionic strength being 0.01. Calf thymus DNA was purchased from Sigma as the highly polymerised sodium salt. Covalently closed circular plasmid DNA, constructed by inserting a 0.9 kilobase fragment into the *Eco*RI site of pBR 322, was a gift from Dr. H.E.D. Lane, Department of Cell Biology, Auckland University. The guanine plus cytosine content of the circular DNA is approx. 50%. DNA concentrations were based on an assumed value for $E_{(P)260}$ of 13200, where the molar extinction coefficient is expressed with respect to nucleotide pairs.

2.2. Viscometry

Measurements using covalently closed circular DNA were performed essentially according to the method of Revet et al. [17] using a Cannon-Ubbelhode series 75 viscometer thermostatically maintained at $20 \pm 0.01°C$ in a Cannon Instrument model MI water bath. The viscometer was fitted with a pair of infrared sensors that auto-

Table 1

Physical properties of di- and triacridines

All compounds analysed correctly for, and have NMR spectra consistent with, the assigned structures. Each compound moves as a single spot on Merck silica gel 60 thin-layer chromatography plates developed in a solvent formed from the top phase of a mixture of *n*-butanol/water/acetic acid in the ratios 5:4:1. Molar extinction coefficients were measured in 0.01 SHE buffer at a ligand concentration of 5 μM using 50-mm quartz cuvettes. Maximum interchromophore distances (*–* distance in fig. 1) are calculated for fully extended linker chains in the staggered conformation using standard bond lengths. The calculations give results in good agreement with measurements made on Corey-Pauling-Koltun molecular models. Note that for the triacridines the values given represent the distance that separates the middle from either of the terminal 9-aminoacridines.

Compound	Formula	Formula weight	Extinction coefficient at 412 nm	Maximal interchromophore distance (Å)
Diacridines				
C2	$C_{35}H_{36}N_6O_2Cl_2$	644	1.72×10^4	15.1
C3	$C_{37}H_{40}N_6O_2Cl_2$	671	1.36×10^4	17.6
C4	$C_{39}H_{44}N_6O_2Cl_2$	700	1.62×10^4	20.0
C5	$C_{41}H_{48}N_6O_2Cl_2$	728	1.57×10^4	22.5
C6	$C_{43}H_{52}N_6O_2Cl_2$	756	1.50×10^4	25.0
C7	$C_{45}H_{56}N_6O_2Cl_2$	784	1.50×10^4	27.5
Triacridines				
C2	$C_{48}H_{45}N_8O_2Cl_3$	872	2.02×10^4	7.5
C3	$C_{50}H_{49}N_8O_2Cl_3$	900	2.13×10^4	8.8
C4	$C_{52}H_{53}N_8O_2Cl_3$	928	2.04×10^4	10.0
C5	$C_{54}H_{57}N_8O_2Cl_3$	957	2.35×10^4	11.3
C6	$C_{56}H_{61}N_8O_2Cl_3$	985	2.26×10^4	12.6
C7	$C_{58}H_{65}N_8O_2Cl_3$	1013	2.08×10^4	13.8

matically trigger an electronic timing device as the meniscus of the solution passes between them, giving a flow time for water of 135 s. Flow times were measured to a precision of ±0.02 s. The viscometer contained 1.0 ml of either a 100 or 200 μM solution of plasmid DNA in 0.01 SHE buffer. Ligand solutions were added in increments of 5–50 μl to a maximum of 250 μl from an Agla precision micrometer syringe via a fine polyethylene tube. After each addition complete mixing was effected by bubbling a current of air down the descending limb of the viscometer. The concentration of the ligand solution being delivered through the plastic tube was assessed before and after each experiment by visible absorption measurements. Solutions were freed of particulate material by passing them through 0.22 μm pore-size Millipore filters before use. No kinetic effects attributable to the rate of complex formation or redistribution of bound ligand between DNA molecules were detected: there was never any consistent trend toward larger or shorter flow times during a set of replicate measurements, even at ligand/DNA ratios where the viscosity was changing rapidly with each increment in ligand concentration. Reduced viscosities were calculated by established methods, taking into account the dilution caused by addition of ligand solutions. For experiments designed to measure the helix extension produced by binding di- and triacridines, calf thymus DNA was sonicated to fragments of molecular weight 2.5×10^5–5×10^5 as previously described [18]. Viscometric measurements on this DNA were performed essentially by the method of Cohen and Eisenberg [19] using the same apparatus described above. In this case, however, because of the smaller reduced viscosity of the sonicated fragments, the DNA concentration was increased to 303 μM. Values of the relative contour length of DNA in the presence of the ligand compared with free DNA, L/L_0, were calculated as the cube root of the measured relative increase in reduced viscosity [11].

3. Results

Fig. 2 shows the effects of ethidium bromide and 9-aminoacridine on the reduced viscosity of covalently closed circular DNA, measurements performed to calibrate the equivalence binding ratio of ethidium for this DNA and to provide a comparison of the helix unwinding angle of 9-aminoacridine with that determined previously [12]. The equivalence input ratios (i.e., molar ratio of total added drug to DNA nucleotide pairs which causes complete relaxation of supercoiling) were found to be 0.073 ± 0.002 (mean of three measurements) and 0.112 ± 0.003 (mean of 2 measurements) for ethidium and 9-aminoacridine, respectively. These input ratios were corrected to binding ratios of 0.072 ± 0.002 for ethidium and 0.107 ± 0.003 for 9-aminoacridine by using equilibrium binding parameters determined for calf thymus DNA in buffers of identical ionic strength [20,21]. This correction assumes that contributions from the free energy of supercoiling to the effective association constant vanish for fully relaxed circles and that the intrinsic binding parameters are indistinguishable for these two DNAs of similar base composition. Taking the helix unwinding angle of ethidium to be 26°C [22] the measurements yield an unwinding angle for 9-aminoacridine of $17.5 \pm 0.5°$, in good agreement with the value of 17.4° determined using PM2 DNA [12]. For comparison with the results of the diacridines and triacridines we have taken the mean value of 17.5° as the unwinding angle of 9-aminoacridine.

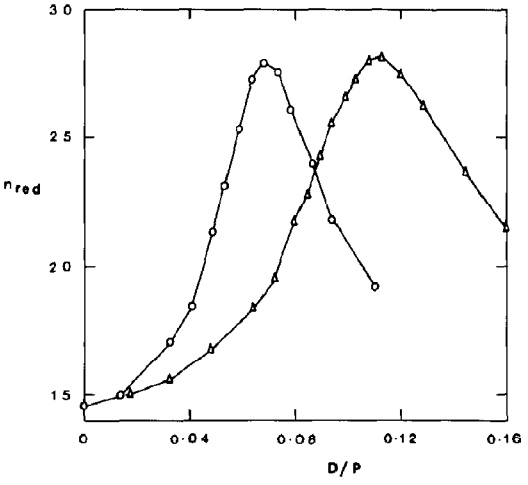


Fig. 2. Effects of ethidium and 9-aminoacridine on the reduced viscosity of covalently closed circular DNA. The initial DNA concentration was 100 μM in nucleotide pairs. The ordinate represents the reduced viscosity in dl/g and the abscissa shows the molar ratio of added drug to DNA base-pairs. (○) Ethidium, (△) 9-aminoacridine.

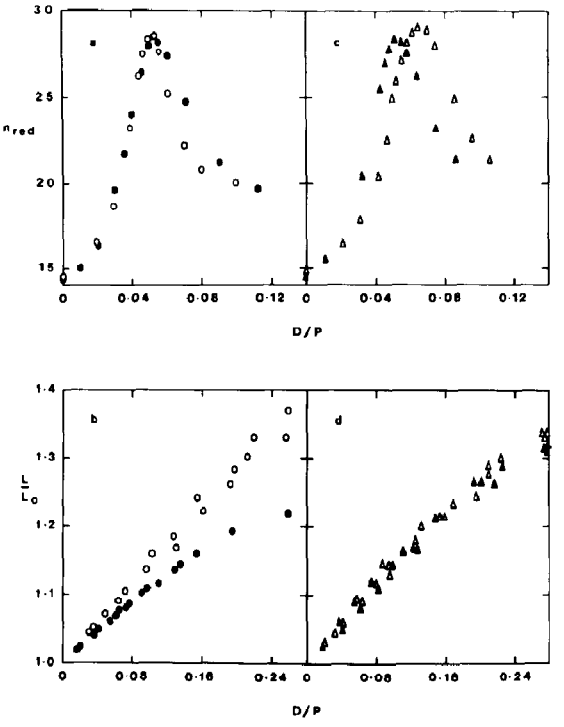


Fig. 3. Interaction of C_2 and C_3 diacridine and triacridine with linear and covalently closed circular DNA. The initial concentrations of covalently closed circular and sonicated calf thymus DNA were 200 and 303 μM in nucleotide pairs, respectively. Panels a and c show the effects of ligands on the reduced viscosity of closed circular DNA. For details of axes see legend to fig. 2. The data presented for each ligand are taken from a single experiment. Panels b and d show the effects of ligands on the relative contour length of sonicated calf thymus DNA fragments. The ordinate represents the calculated ratio of contour lengths in the presence (L) or absence (L_0) of ligand. The abscissa represents the molar ratio of added drug to DNA base-pairs. The data presented for each ligand are derived from two independent experiments. (○) C_2 diacridine, (●) C_2 triacridine, (△) C_3 diacridine, (▲) C_3 triacridine.

Helix unwinding angles for the polyfunctional ligands were determined by titration of the supercoiling at DNA concentrations of 100 and 200 μM, measurements being made at least twice, frequently three times, at both concentrations. For each compound studied the equivalence input ratio was found to be indistinguishable at the two DNA concentrations, indicating that all of the added ligand was bound at equivalence. This finding is expected for ligands of this type which have association constants in excess of 10^6 M^{-1} under these experimental conditions [14,15]. The extension of the DNA helix associated with binding of triacridines and diacridines was investigated by using sonicated rod-like fragments of calf thymus DNA. Plots were constructed of the relative increase in contour length, L/L_0, as a function of ligand/nucleotide pair input ratio and the least-squares slopes of the linear portions (generally speaking up to D/P values of 0.15–0.20) evaluated. Representative plots of the effects of the polyfunctional ligands on the reduced viscosity of closed circular DNA, and of their effects on the helix extension of linear DNA are shown in figs. 3–5. Values of their helix unwinding angles and increments in DNA contour length are recorded in table 2.

The C_2, C_3 and C_4 triacridines have central-to-terminal interchromophore distances that provide room for only a single base-pair between each chromophore, assuming a DNA-ligand complex in which the DNA helix is not severely bent (table 1). For a similar, minimally distorted, complex the largest of the corresponding diacridines has a max-

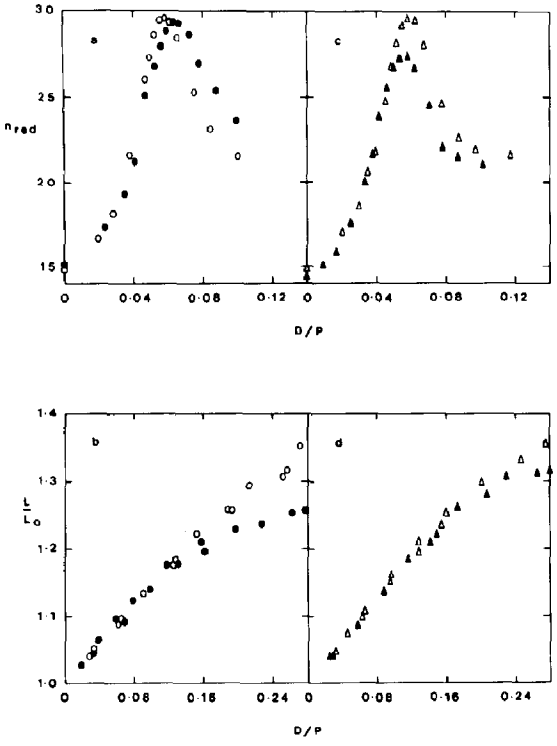


Fig. 4. Interaction of C_4 and C_5 diacridine and triacridine with linear and covalently closed circular DNA. For details see legend to fig. 3. (○) C_4 diacridine, (●) C_4 triacridine, (△) C_5 diacridine, (▲) C_5 triacridine.

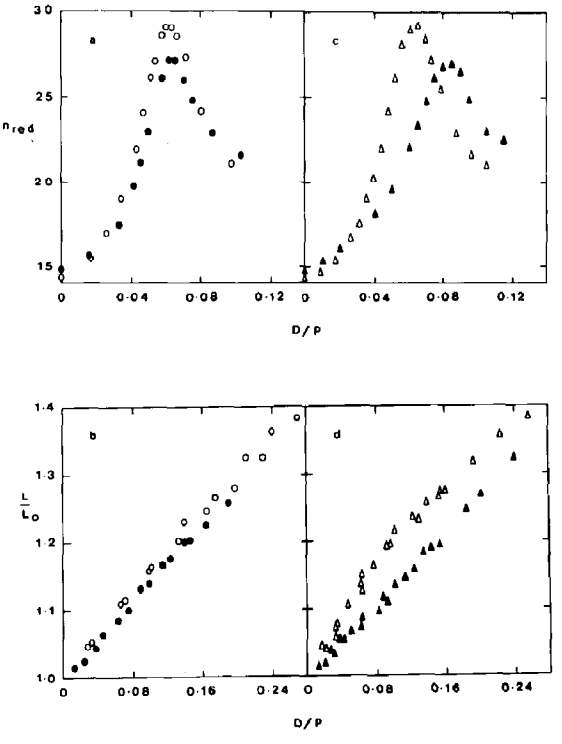


Fig. 5. Interaction of C_5 and C_7 diacridine and triacridine with linear and covalently closed circular DNA. For details see legend to fig. 3. (○) C_6 diacridine, (●) C_6 triacridine, (△) C_7 diacridine, (▲) C_7 triacridine.

Table 2

Interaction of di- and triacridines with linear and covalently closed circular DNA

The equivalence binding ratio is expressed as the number of ligand molecules bound per base-pair and is the mean of the values measured at DNA concentrations of 100 and 200 μM. The helix unwinding angle is calculated taking that of ethidium to be 26° [22]. The slope of the helix extension plot is defined with respect to nucleotide pairs, and the increase in contour length is calculated assuming that a gradient of 1 is equivalent to an extension of 3.4 Å. The relative unwinding angle is the ratio of the ligand unwinding angle to that of 9-aminoacridine (17.5°) and, similarly, the relative helix extension is the ratio of the helix extension produced by binding of the ligand to that produced by 9-aminoacridine (the slope of the L/L_0 plot for the latter is 0.90 when expressed in base-pair units, taken from ref. 12). All measurements were made in 0.01 SHE buffer at 20°C.

Compound	Equivalence binding ratio	Helix unwinding angle (°)	Slope of helix extension plot	Increase in contour length (Å)	Relative unwinding angle	Relative helix extension
Diacridines						
C2	0.0530±0.0015	35.3±1.0	1.40±0.05	4.8±0.2	2.02	1.56
C3	0.0656±0.0018	28.5±0.8	1.49±0.06	5.1±0.2	1.63	1.68
C4	0.0570±0.0012	32.8±0.7	1.45±0.06	5.0±0.2	1.87	1.61
C5	0.0596±0.0016	31.4±0.8	1.61±0.07	5.5±0.2	1.79	1.79
C6	0.0610±0.0018	30.7±0.9	1.60±0.07	5.4±0.2	1.75	1.79
C7	0.0630±0.0014	29.7±0.7	2.21±0.09	7.5±0.3	1.70	2.46
Triacridines						
C2	0.0560±0.0012	33.4±0.7	1.12±0.05	3.8±0.2	1.91	1.25
C3	0.0500±0.0011	37.4±0.8	1.46±0.06	5.0±0.2	2.14	1.61
C4	0.0610±0.0014	30.7±0.7	1.51±0.06	5.1±0.2	1.75	1.69
C5	0.0580±0.0013	32.3±0.7	1.56±0.07	5.3±0.2	1.85	1.74
C6	0.0640±0.0012	29.3±0.6	1.41±0.06	4.8±0.2	1.67	1.58
C7	0.0850±0.0019	22.0±0.5	1.32±0.06	4.5±0.2	1.26	1.49

imal interchromophore distance that permits it to span up to 3 or 4 base-pairs (see table 1). Helix unwinding angles for the C_2–C_4 tri- and diacridines, which fall in the range 28.5–37.4° (table 2), are typical of those found for other diacridines [2,11–13] and are thus characteristic of bifunctional reaction. The reduced viscosities of the fully-relaxed circular DNA complexes of these compounds (28–29.5 dl/g) are not distinguishable from each other nor from those of ethidium and 9-aminoacridine (see figs. 2, 3a and c and 4a), indicating no peculiar structural perturbations to the DNA helix. Increases in DNA contour length accompanying binding of this set of di- and triacridines support the notion that these ligands are bifunctional. With the exception of that for C_2 triacridine the slopes of the helix extension plots for the C_2–C_4 compounds are experimentally indistinguishable, centering on a value of approx. 1.45 (table 2). Similarly, the shapes of the extension plots for the C_3 pair of di- and triacridines are practically indistinguishable with the relative increase in contour length reaching limiting values of 1.34 and 1.31, respectively, for input ratios greater than 0.28 (fig. 3d, measurements extend to $D/P = 0.45$ but data for largest input ratios are not shown). By contrast, the plots for the matched pairs of the C_2 and C_4 ligands are more dissimilar. That for the C_2 diacridine is linear to the highest input ratio studied ($D/P_{max} = 0.26$, $L/L_{0\,max} = 1.37$) whereas that for C_2-triacridine has an unusually small slope with L/L_0 reaching a plateau value of 1.22 at input ratios of 0.26 and above (fig. 3b, data extend to $D/P = 0.40$). For the C_4 di- and triacridines the maximum relative extension reaches values of 1.36 and 1.28, respectively, at input ratios in the range 0.28–0.45 (fig. 4b, data for highest input ratios not shown). Extrapolating the linear portion of the helix extension plots to the observed maximum increase in relative contour length suggests that for the C_2 and C_4 diacridines the DNA saturates with 1 ligand molecule bound

per 4 base-pairs, whereas for the triacridines the binding site size increases to 5 base-pairs. This conclusion also extends to C_3 triacridine but the apparent site size of C_3 diacridine falls midway between 4 and 5 nucleotide pairs.

The C_5, C_6 and C_7 triacridines have the capacity to encompass two base-pairs in each arm of a linear trisintercalated complex and the largest of the corresponding diacridines has the potential to cover up to a maximum of 4 to 5 base-pairs, assuming no pronounced bending of the DNA backbone and allowing for helical twist and local unwinding (table 1). Helix unwinding angles of the C_5–C_7 group of diacridines are again typical of bifunctional reaction, their range of 29.7–31.4° being 1.70–1.79-times that of 9-aminoacridine. Similarly, the unwinding angles of C_5 and C_6 triacridine are also characteristic of bisintercalation whereas the value for C_7 triacridine is anomalously low (table 2). It is interesting to note that the reduced viscosity of the fully relaxed circular DNA-triacridine complexes is consistently 2–2.5 dl/g lower than that of the diacridine complexes for the C_5–C_7 compounds (fig. 4c, 5a and c). The finding that these larger triacridines fail to trisintercalate is supported by their effects on the contour length of sonicated DNA. Binding of the C_5 and C_6 triacridines causes a similar helix extension to that accompanying binding of the corresponding diacridines, the slopes of the L/L_0 plots being 1.58–1.79-times that of 9-aminoacridine (figs. 4a and 5a, and table 2). The helix extension data have been measured for input ratios up to 0.45 for the pair of C_5 ligands (values greater than 0.28 not shown), again leading to estimates of 4 and 5 base-pairs, respectively, for the binding site size of the diacridine and triacridine. Whilst the extension data for the C_6 di- and triacridines are confined to those points shown in fig. 5b, they too are consistent with this general trend in stoichiometry. The effects of the C_7 diacridine and triacridine on DNA contour length are somewhat different compared to those of their lower homologues. The L/L_0 plot for the triacridine has a perceptibly smaller slope and is linear to input ratios of 0.24, the highest value studied (fig. 5d). For C_7 diacridine the initial slope is the largest amongst all the compounds investigated here and the data are much more curvilinear than is typically observed (fig. 5d).

4. Discussion

The most significant finding arising from the present work is the failure of the largest members of the triacridine series to trisintercalate, notwithstanding the fact that experiments with space-filling molecular models indicate no geometrical impediments to trifunctional reaction for those homologues (i.e., C_6 and C_7 triacridine) capable of comfortably spanning two base-pairs between each chromophore. Indeed, these same model-building studies reveal no geometrical reasons for the lack of trifunctional reaction for the C_3 and C_4 triacridines which can encompass only a single base-pair in each arm of the complex. Thus, it follows that there must be energetic constraints, not evident with simple molecular models, which prevent trisintercalation for this ligand system. It seems likely, at least for those compounds capable of forming 'two base-pair sandwich' complexes, that such constraints will be largely associated with the requirement that all three chromophores adopt conformations suitable for intercalative binding without transmitting unfavourable distortions along the ligand backbone which might otherwise prevent intercalation of neighbouring acridines.

That the relationship between the flexibility of the linker chain and the conformational freedom of the middle acridine ring may be a crucial determinant of the energetics of trisintercalation is foreshadowed by comparison of our findings with those of Gaugain et al. [15] and Hansen et al. [16]. The triacridine of Gaugain et al. [15] differs from our C_7 triacridine only by the inclusion of an amide group in the variable portion of each arm of the ligand, thereby increasing the maximum central-to-terminal interchromophore distance by 3.6 Å. The middle acridine in both compounds may be viewed as an *N*-acridinyl derivative of 3-aminoglutaric acid, and space-filling molecular models suggest that this chromophore has limited conformational freedom as a result of steric interactions with the linker chain. It is interesting to

note, therefore, that increasing the central-to-terminal interchromophore distance by 3.6 Å in a bisintercalating triacridine which is already large enough geometrically to span two base-pairs between each chromophore (i.e., the C_7 triacridine), sufficiently relaxes the conformational constraints on the ligand backbone to permit trifunctional reaction [15]. By contrast, attention is drawn to the significance of relaxing conformational strain around the central chromophore by comparision with the work of Hansen et al. [16]. The maximum end-to-end interchromophore distance of these authors' triacridine is 17.6 Å, a value identical to that of the C_3 di- and triacridines, and the central chromophore is attached by a propyl chain to the ligand backbone [16]. Insertion of the propyl linker provides sufficient degrees of freedom to allow intercalation of all three acridine rings in what must be, in the absence of bending or kinking of the DNA, a 'one base-pair sandwich' trisintercalated complex. In addition, it should be noted that this triacridine contains a protonated tertiary amine in the middle of its backbone to which the propyl chain is connected, so that the net charge on the molecule is +4 at neutral pH [23]. This extra charge may provide an increase in the free energy of binding, a portion of which may assist in overcoming conformational barriers in both the ligand and the DNA backbones that might otherwise inhibit trisintercalation in a manner that doubly violates the 'neighbour exclusion' mode of binding [1,2,11–13].

With the exceptions of C_3 and C_7 triacridine the helix unwinding angles of the polyfunctional ligands studied here are typical of simple alkyl-linked and amide-containing diacridines [11,13,15], these values being lower than those found for the polyamine-linked diacridines [2,12]. In contrast, however, aside from those of C_7 diacridine and C_2 and C_7 triacridine, values for the increments in DNA contour length accompanying binding are characteristic of the lower extensions observed for the polyamine-linked dimers [12]. The low unwinding and extension parameters for C_7 triacridine, together with the very curvilinear shape of the extension plot (fig. 5d) suggest that this compound may bind in a mixed bifunctional/monofunctional mode. The C_7 diacridine stands alone as the only compound studied which has a helix extension greater than double that of 9-aminoacridine, being in fact exactly twice the value found for 9-methylaminoacridine [12]. Whilst the low value for the helix extension of C_2 triacridine may be taken as evidence for bending or kinking of the DNA helix (see refs. 11 and 12), no peculiarities were observed for the interaction of this ligand with closed circular DNA to support this conclusion. By contrast, evidence for structural differences between complexes of the C_5, C_6 and C_7 di- and triacridines is provided by the finding of lower reduced viscosities at equivalence for circular DNA-triacridine complexes. This observation suggests that DNA complexes of the larger triacridines are less stiff or more bent than those of the corresponding diacridines (see refs. 11 and 12). Interestingly, this difference between the di- and the triacridines is not detectable for the shorter homologues in each series.

It is a moot point as to which two of the three chromophores of the triacridines actually intercalate. By analogy with the simple alkyl-linked diacridines the linker in C_2 triacridine is too short to permit simultaneous intercalation of the central and a terminal acridine which implies that bisintercalation of this compound involves the two terminal chromophores. The same analogy suggests there is sufficient room to encompass a single base-pair between the central and terminal acridines for C_3 and C_4 triacridine. However, a cautionary note is sounded by the findings of King et al. [13] that diacridines with more rigid amide-containing linkages require interchromophore distances greater than 8.8 Å for bifunctional reaction. The C_5, C_6 and C_7 triacridines are sufficiently large for a terminal and the central chromophore to span two base-pairs in a bisintercalated complex, thus making bisintercalation of these chromophores a more likely proposition. Whilst there are presently insufficient data to define which of the chromophores is intercalated for C_3 triacridine and higher homologues, it is possible that the diminished reduced viscosity of the relaxed-circular DNA complexes of the C_5–C_7 triacridines betokens a change in binding mechanism, whereby bisintercalation of the terminal chromophores of C_2, C_3 and C_4 triacridine gives way to bifunctional

reaction of juxtaposed chromophores for the larger members in the series.

The discrepancy between the conclusion that C_3 triacridine, although exhibiting the largest unwinding angle in the series, still intercalates bifunctionally and the report [14] which finds this ligand to be trifunctional, is related to the measured values of the helix unwinding angles of 9-aminoacridine and the triacridine. Both reports concur on the value of the unwinding angle of C_3 diacridine (28.5 and 29°) but Atwell et al. [14] underestimate the unwinding angle of 9-aminoacridine by approx. 15% compared to that found here and elsewhere [12]. Additionally, while the unwinding angle of the C_3 triacridine is significantly greater than that of either the C_3 diacridine, in both studies, or the corresponding diacridine bearing a point charge instead of a third central chromophore [14], the previous work determined a value approx. 20% greater than that found here. That such relatively small differences in measured unwinding angles can result in very different conclusions emphasises the importance of assessing the mode of action of polyfunctional intercalators by as many methods as possible. Indeed, Gaugain et al. [15] have already pointed to the difficulties and discrepancies that may be encountered with complex triacridines using what are normally considered definitive methods. One approach to help solve these problems is to study homologous series of polyfunctional ligands so that added credence may be given to the results by identification of consistent trends in the structure-function relationships.

Acknowledgements

This work was supported by grants from the Auckland Division of the Cancer Society of New Zealand, the Medical Research Council of New Zealand and the Australian Research Grants Scheme.

References

1 M.J. Waring and L.P.G. Wakelin, Nature 252 (1974) 653.
2 J.-B. LePecq, M. LeBret, J. Barbet and B. Roques, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 2915.
3 T.K. Chen, R.M. Fico and E.S. Canellakis, J. Med. Chem. 21, (1978) 868.
4 B.F. Cain, B.C. Baguley and W.A. Denny, J. Med. Chem. 21, (1978) 658.
5 K.F. Kuhlmann, N.J. Charbeneau and C.W. Mosher, Nucleic Acids Res. 5, (1978) 2629.
6 L.P.G. Wakelin, T.S. Creasy and M.J. Waring, FEBS Lett. 104, (1979) 261.
7 N. Capelle, J. Barbet, P. Dessen, S. Blanquet, B. Roques and J.-B. LePecq, Biochemistry 18, (1979) 3354.
8 D. Pelaprat, A. Delbarre, I. LeGuen, B.P. Roques and J.-B. LePecq, J. Med. Chem. 23, (1980) 1336.
9 K.F. Kuhlmann and C.W. Mosher, J. Med. Chem. 24 (1981) 1333.
10 K. Dawson, Cancer Res. 43, (1983) 2880.
11 L.P.G. Wakelin, M. Romanos, T.K. Chen, D. Glaubiger, E.S. Canellakis and M.J. Waring, Biochemistry 17, (1978) 5057.
12 R.G. McR. Wright, L.P.G. Wakelin, A. Fieldes, R.M. Acheson and M.J. Waring, Biochemistry 19, (1980) 5825.
13 H.D. King, W.D. Wilson and E.J. Gabbay, Biochemistry 21, (1982) 4982.
14 G.J. Atwell, W. Leupin, S.J. Twigden and W.A. Denny, J. Am. Chem. Soc. 105, (1983) 2913.
15 B. Gaugain, J. Markovits, J.-B. LePecq and B. Roques, FEBS Lett. 169, (1984) 123.
16 J.B. Hansen, T. Koch, O. Buchardt, P.E. Nielsen, B. Norden and M. Wirth, J. Chem. Soc. Chem. Commun. (1984) 509.
17 B.M.J. Revet, M. Schmir and J. Vinograd, Nat. New Biol. 229, (1971) 10.
18 L.P.G. Wakelin and M.J. Waring, Biochem. J. 157, (1976) 721.
19 G. Cohen and H. Eisenberg, Biopolymers 8, (1969) 45.
20 J.-B. LePecq and C. Paoletti, J. Mol. Biol. 27, (1967) 87.
21 W.R. Wilson, B.C. Baguley, L.P.G. Wakelin and M.J. Waring, Mol. Pharmacol. 20, (1981) 404.
22 J.C. Wang, J. Mol. Biol. 89, (1974) 783.
23 J.B. Hansen and O. Buchardt, J. Chem. Soc. Chem. Commun. (1983) 162.